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Turkey Origin Reovirus-Induced Immune Dysfunction in Specific Pathogen Free and Commercial Turkey Poults

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SUMMARY. Recently, pathogenesis studies, using genetically distinct turkey-origin reoviruses (TRVs), revealed that poults infected with certain TRV isolates had moderate to severe bursal atrophy, suggesting virus-induced immune dysfunction. In order to characterize the effect of TRV infection on the turkey immune system, classical assays were undertaken to quantify the humoral and cell-mediated immune responses in small Beltsville and broad-breasted white poults infected with the TRV isolate NC/SEP-R44/03. A marked effect on the cutaneous basophil hypersensitivity response, and on the antibody response to Newcastle disease virus (NDV) exposure, was noted in commercial and specific pathogen free (SPF) poults inoculated with NC/SEP-R44/03 at three days of age. Moderate to severe bursal atrophy, similar to that noted previously in SPF poults, occurred in commercial poults inoculated at three days of age. This immune dysfunction and bursal atrophy was not present in commercial poults inoculated at three weeks of age.

RESUMEN. Disfunción del sistema inmune inducida por reovirus de pavos, en pavitos comerciales y libres de patógenos específicos.

Estudios recientes de patogénesis usando reovirus genéticamente diferentes originados en pavos, revelaron que los pavitos infectados con cierto tipo de estos aislados presentaban atrofia de moderada a severa de la bolsa de Fabricio, sugiriendo que estos virus inducen disfunción del sistema inmune. Con el fin de caracterizar los efectos de estos reovirus sobre el sistema inmune de pavos, se llevaron a cabo pruebas clásicas para cuantificar las respuestas inmunes tanto humoral como mediada por células en pavos pequeños Beltsville y pavos blancos de doble pechuga infectados con el aislado de reovirus de pavo identificado como NC/SEP-R44/03. En pavitos comerciales y en libres de patógenos específicos inoculados a los tres días de edad, se observó un efecto marcado en la hipersensibilidad cutánea basofílica y en la respuesta de anticuerpos a la exposición con el virus de la enfermedad de New Castle. También se observó de moderada a severa atrofia de la bolsa de Fabricio en pavitos comerciales, similar a la observada previamente en pavitos libres de patógenos específicos inoculados a los tres días de edad. Esta disfunción del sistema inmune y atrofia de la bolsa de Fabricio no se observaron en pavitos comerciales inoculados a las tres semanas de edad.

Key words: avian reovirus, immunosuppression, enteric virus

Abbreviations: CBH = cutaneous basophile hypersensitivity; DPI = days postinoculation; EID = egg infectious dose; NDV = Newcastle disease virus; PEC = poult enteritis complex; PEMS = poult enteritis mortality syndrome; PHA-P = phytohemagglutinin-P; RSS = runting-stunting syndrome; SEPRL = Southeast Poultry Research Laboratory; S/P = sample to positive (ratio); SPF = specific pathogen free; TRV = turkey reovirus

The avian reoviruses are an economically important group of pathogens that have been implicated in, or associated with, a number of disease states in birds. These conditions are, in many cases, multifactorial enteric syndromes that involve other etiologic agents such as runting-stunting syndrome (RSS) in broilers or poult enteritis complex (PEC) and poult enteritis mortality syndrome (PEMS) in turkeys. Further, the avian reoviruses have been implicated in several non-enteric diseases of poultry, including tenosynovitis, a condition with amply demonstrated reovirus etiology (4,5,15,22). Nonetheless, most avian reovirus infections remain asymptomatic (1). Recently, several novel turkey-origin reoviruses (TRVs) have been described based upon their pathogenesis in turkeys and chickens (20), the unique sequences of their S1 and S3 genome segments (3,6,16), and their tissue distribution in infected poults (11). Two of these TRVs, isolates NC/SEP-R44/03 and NC/98, caused moderate to severe bursal atrophy in young poults, a condition that can result in permanent immunosuppression (11,14,20). In order to characterize the extent of the immune dysfunction caused by a TRV in commercial and specific pathogen free (SPF) poults, experiments were designed to investigate the humoral and cell-mediated immune response in experimentally infected birds. The effect of TRV exposure on poults was evaluated using a cutaneous basophil hypersensitivity assay and by determining their antibody response to Newcastle disease virus (NDV). Further, the effect of TRV infection on poult lymphoid tissue was evaluated by histopathologic examination.

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MATERIALS AND METHODS

Turkey origin reovirus. The TRV isolates were originally obtained from commercial turkey flocks experiencing enteric disease signs (6,16,20). The TRV isolate NC/SEP-R44/03 was isolated in Vero cells from the pooled intestinal contents of poults with enteric disease (20). NC/SEP-R44/03 was propagated for no more than four passages in Vero cells in order to exclude the turkey astroviruses and the turkey coronavirus. The absence of these agents was confirmed via real-time reverse transcriptase polymerase chain reaction, as previously described (19).

Experimental infection with TRV NC/SEP-R44/03. Broad-breasted white poults were obtained at three days of age from a commercial hatchery (Privett Hatchery, Portales, NM), and SPF small Beltsville white poults were obtained from in-house flocks at the Southeast Poultry Research Laboratory (SEPRL). The birds were individually tagged and

divided into six groups of 10. Two identical experiments were performed with the commercial breed; one in which birds were inoculated at three days posthatch and one in which birds were inoculated at three weeks posthatch. One experiment was also performed with the SPF poults, in which the birds were inoculated at three days posthatch. Each group that received TRV was inoculated with $10^{3.5}$ ($TCID_{50}$) of the TRV isolate NC/SEP-R44/03 (20) in 0.2 ml cell culture supernatant, by the oral route with an oral gavage needle, and housed in Horsfal isolators with *ad libitum* access to feed and water. One group in each experiment (three groups total) received 0.2 ml sterile 50% DMEM/50% F12 medium and served as sham-inoculated controls. At days postinoculation (DPI) 2, 4, 7, 11, and 14, all poults were weighed, cloacal swabs were collected from five birds in each group, and two birds from each group were euthanatized in a CO_2 chamber in accordance with SEPRL's Institutional Animal Care and Use Committee guidelines and examined postmortem.

Histopathology. At 8 and 15 DPI, two birds from each group were euthanatized and the proventriculus, cecal tonsils, duodenum/pancreas, jejunum, ileum, liver, spleen, thymus, and bursa of Fabricius were collected and fixed in 10% neutral buffered formalin, paraffin embedded, and stained with hematoxylin and eosin, by standard methods, for subsequent microscopic evaluation.

Cutaneous basophil hypersensitivity assay (CBH). Evaluation of the CBH was performed as previously described, and time points for measurements were selected based upon reported results with young chickens (2). The procedure was identical for poults inoculated at three days and three weeks of age. At nine and 16 DPI, 10 poults from the sham-inoculated group and 10 poults from the NC/SEP-R44/03-inoculated experimental group were chosen for the assay. Prior to injection, the thickness of the toe web between the third and fourth digits of both feet of each bird was measured using a positive pressure digital micrometer (Mitutoyo Corporation, Kawasaki, Japan). The premeasured toe web of the left foot of each bird was injected intradermally with 200 μg phytohemagglutinin-P (PHA-P) (Sigma, St. Louis, MO) in 100 μl sterile PBS; the premeasured toe web of the right foot of each bird was injected with 100 μl sterile PBS. The postinjection thickness was measured, as before, at 12 and 24 hr postinjection. The CBH response was calculated as ([postinjection toe web thickness, left foot] – [preinjection toe web thickness, left foot]). The mean CBH response for birds in sham and inoculated groups was analyzed using a *t*-test (GraphPad Prism 5, GraphPad Software, San Diego, CA).

Evaluation of antibody response to NDV. At 1 DPI, 10 poults inoculated with NC/SEP-R44/03 and 10 sham-inoculated poults were inoculated, by the ocular route, with 10^5 EID_{50} of the LaSota strain of NDV obtained from the SEPRL repository. At 21 DPI, approximately 2 ml of blood were collected from each poult and each poult was re-inoculated with the LaSota strain of NDV, as described above. At 42 DPI, approximately 2 ml of blood were collected from each poult, and the poults were euthanatized in a CO_2 chamber in accordance with Institutional Animal Care and Use Committee guidelines. Antibody titers to NDV were determined using a commercial ELISA kit (IDEXX, Westbrook, ME) according to the manufacturer's instructions.

RESULTS

Cutaneous basophil hypersensitivity assay. The mean CBH response in SPF and commercial poults inoculated at three days of age with TRV was significantly reduced ($P < 0.05$) compared to the sham-inoculated groups at all time points postinjection, with the exception of SPF poults evaluated at 12 hr post-PHA-P inoculation at 9 DPI (Fig. 1). All poults injected with PHA-P had visibly thickened skin between the third and fourth digits of the left foot at the 12- and 24-hour time points. After the injection of sterile PBS, there was no significant thickening of the skin, noted visibly or via micrometer measurement, between the third and fourth digits of the right foot of each poult. Commercial poults inoculated with NC/SEP-R44/03 at three weeks of age and assayed at 9 and 15 DPI showed no significant reduction in the CBH response compared to sham-inoculated poults (data not shown). Data sets with less than 10 poults are the result of nonspecific mortality that did not appear to be due to TRV infection.

Evaluation of antibody response to NDV exposure. The IDEXX NDV antibody test kit for turkeys was used to determine the relative level of antibody to NDV in serum by calculating the sample to positive (S/P) ratio. In SPF poults inoculated with NC/SEP-R44/03 at three days of age, the mean ($n = 10$) S/P ratio at 21 days post-NDV exposure was 0.11 compared to 1.18 in the sham-inoculated poults ($n = 10$) (Table 1). Further, only one of the 10 serum samples from NDV inoculated, reovirus-infected poults was positive for the presence of NDV antibodies (S/P ratio of >0.2). Nine of the 10 sham-inoculated poults had an S/P ratio of >0.2. At 42 days post-NDV exposure (which included an NDV boost at 21 days), the sham-inoculated mean S/P ratio ($n = 10$) was 0.98, compared to 0.50 for the TRV-inoculated group ($n = 8$). All of the sham-inoculated poults were positive for NDV antibodies, while four of eight of the TRV-inoculated birds had S/P ratios >0.2. Results were similar for the commercial poults inoculated with NC/SEP-R44/03 at three days of age, with 21-day post-NDV exposure S/P ratios of 0.81 for sham-inoculated poults ($n = 7$) and 0.12 for TRV-inoculated poults ($n = 9$). The trend continued at 42 days post-NDV exposure, with S/P ratios of 0.74 for the sham inoculates ($n = 7$) and 0.46 for the TRV-inoculated poults ($n = 8$). In commercial poults that were inoculated with NC/SEP-R44/03 at three weeks of age, there was little difference in the S/P ratios when compared to the sham inoculates at both the 21- and 42-day post-NDV exposure time points (see Table 2). All poults (both sham- and TRV-inoculated groups) were positive for NDV antibodies 42 days post-NDV exposure, while 67% of the sham-inoculated poults and 100% of the TRV-inoculated poults were positive at 21 days post-NDV exposure (Table 2).

Histopathology. Poults inoculated with reovirus isolate NC/SEP-R44/03 at three days of age had moderate to severe bursal atrophy at 8 and 15 DPI (Fig. 2), similar to the atrophy observed previously in SPF poults (11,20). Both the cortical and medullary regions of the bursal follicles were affected. Fibroplasia was present surrounding the bursal follicles. Also, mild to moderate lymphoid depletion in the spleen peri-ellipsoidal and peri-arteriolar sheaths, coupled with histiocytic and ellipsoidal hyperplasia, was observed at 8 DPI. Mild lymphocytic infiltration in the submucosa was observed in the jejunum, duodenum, and ceca at 8 and 15 DPI. Mild hyperplasia of the peripheral pancreatic lymphoid tissue was observed. In the proventriculus, mild lymphoid infiltration was present in the lamina propria of the mucosa and in the proventricular glands. No lesions were present in the thymus, liver, or gizzard. No microscopic lesions were observed, at any time, in tissues collected from sham-inoculated poults.

DISCUSSION

Previous observations that experimental infection of poults with TRVs caused moderate to severe atrophy of the bursa of Fabricius (20), perhaps as the result of virally-induced apoptosis (11), suggest that TRV infection may cause immune dysfunction in turkeys. During an experimental infection, TRV antigen and nucleic acid can be detected in the bursa of Fabricius, spleen, and enterocytes, via immunohistochemistry and *in situ* hybridization; and further circumstantial evidence for immune dysfunction in TRV-infected poults includes evidence of apoptosis in the spleen and virus-induced bystander apoptosis in lymphocytes (11). The present study was

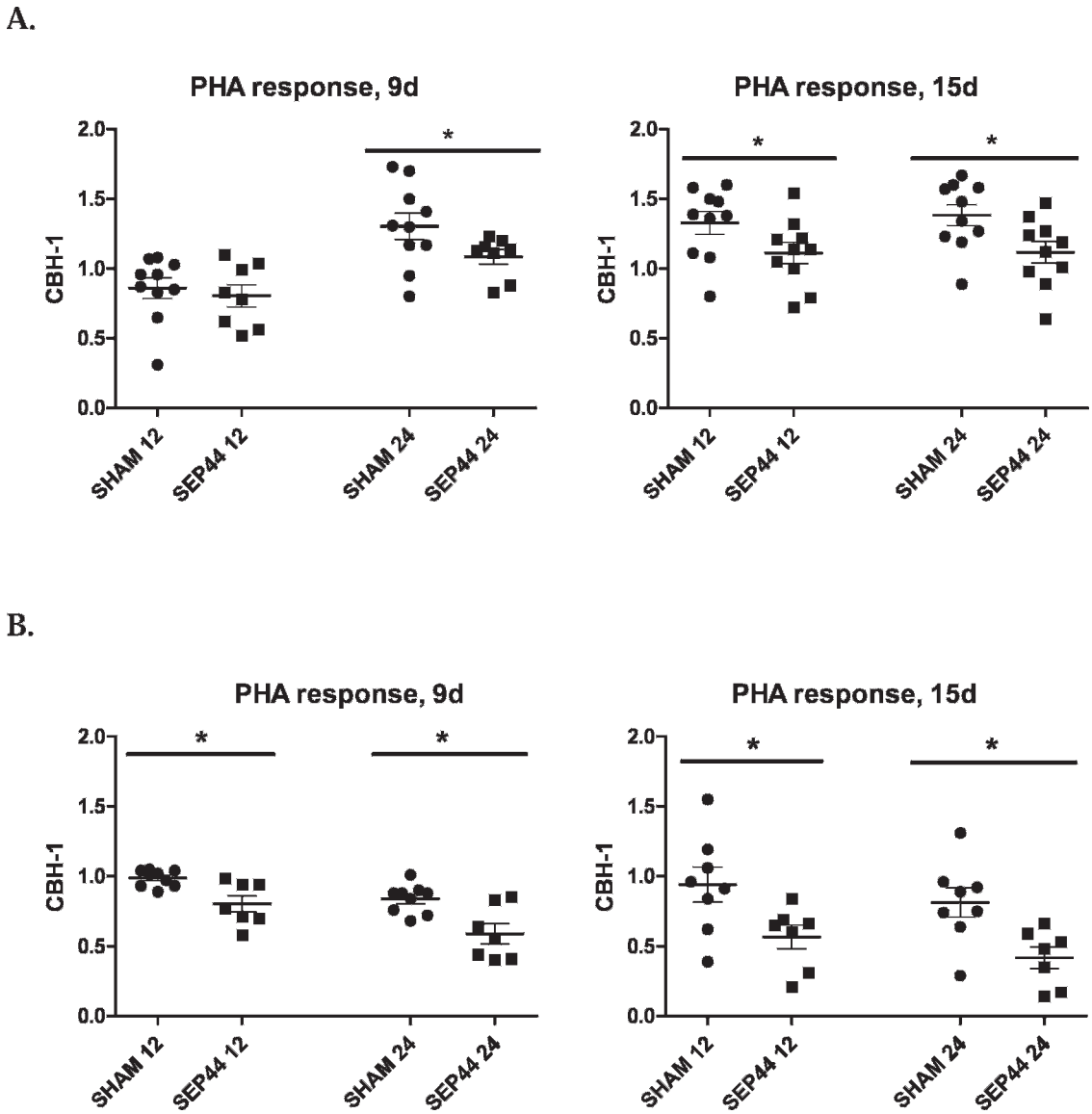

Fig. 1. (A) Graph comparing the CBH response (in mm) in virus- and sham-inoculated 3 day old SPF turkey poults at 9 and 15 DPI. The interdigital skin thickness of each poult was measured at 12 and 24 hr after an injection of the mitogen PHA-P. (B) Graph comparing the CBH response in virus- and sham-inoculated 3-day-old commercial turkey poults at 9 and 15 DPI. The interdigital skin thickness of each poult was measured at 12 and 24 hr after an injection of the mitogen PHA-P. Bars represent the mean interdigital skin thickness and the standard error of the mean.

Table 1. Summary of the antibody levels to the La Sota NDV strain in SPF and commercial poults either sham-inoculated (NDV only) or inoculated with the TRV strain NC/SEP-R44/03 (NDV + SEP44) at 3 days of age.

	NDV antibody data, SPF poults			
	21 days post-NDV		42 days post-NDV	
	NDV only	NDV + SEP44	NDV only	NDV + SEP44
Mean S/P ratio	1.18	0.11	0.98	0.50
#pos/#neg	9/1	1/9	10/0	4/4
% positive	90	10	100	50
	NDV antibody data, commercial poults			
	21 days post-NDV		42 days post-NDV	
	NDV only	NDV + SEP44	NDV only	NDV + SEP44
Mean S/P ratio	0.81	0.12	0.74	0.46
#pos/#neg	4/3	1/8	5/2	7/1
% positive	57	11	71	88

Table 2. Summary of the antibody levels to the La Sota NDV strain in commercial poults either sham-inoculated (NDV only) or inoculated with the TRV strain NC/SEP-R44/03 (NDV + SEP44) at 3 weeks of age.

	21 days post-NDV		42 days post-NDV	
	NDV only	NDV + SEP44	NDV only	NDV + SEP44
Mean S/P ratio	0.99	1.12	1.56	1.40
#pos/#neg	6/3	10/0	9/0	10/0
% positive	67	100	100	100

designed to quantify the immune dysfunction caused by a TRV, by determining the effect of TRV infection on the humoral and cell-mediated immune responses, using some of the available tests for turkeys which evaluate a systemic response.

The cell-mediated hypersensitivity of poults was determined using the T-cell mitogen phytohemagglutinin-P (PHA-P). The intradermal injection of PHA-P in poultry resulted in the infiltration of basophils and non-granulated mononuclear cells, such as lymphocytes and macrophages, into the injection site, resulting in a grossly visible, measurable inflammation (2,21). Interestingly, the reduction in the CBH response in TRV-infected SPF and commercial poults was not accompanied by notable lesions in the thymus. Impaired *in vitro* peripheral blood lymphocyte responses to PHA-P during avian reovirus infections in chickens have been reported (9,10). Similarly, the authors of an earlier study did not observe any microscopic lesions in the thymus of infected birds (9). Other evidence in chickens suggests that reovirus-activated macrophages may serve to suppress certain T-cell functions during an infection, subsequently inhibiting the action of an introduced mitogen (12,13,17). The apoptosis noted in the bursa of Fabricius of TRV-infected poults in a

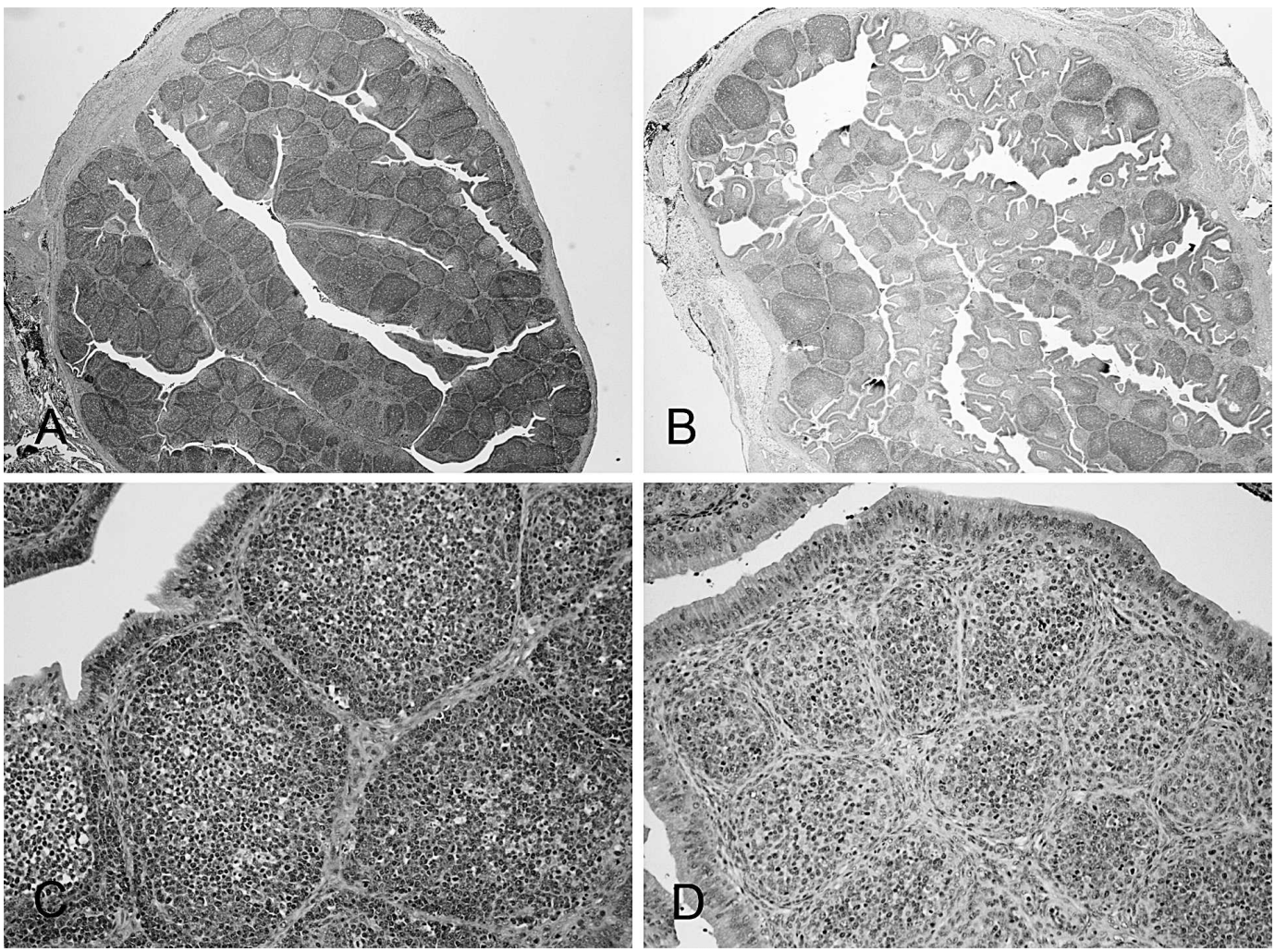

Fig. 2. (A) Photomicrograph of the bursa of Fabricius of a sham-inoculated commercial poult; hematoxylin-eosin staining (HE); HE = 20×. (B) Photomicrograph at 7 days DPI of the bursa of Fabricius of a commercial poult inoculated at three days of age with turkey-origin reovirus (TRV) isolate NC/SEP-R44/03 (SEP/44). Bursal atrophy with follicle lymphoid depletion and stromal fibroplasia; HE = 20×. (C) Photomicrograph of the bursa of Fabricius of a sham-inoculated commercial poult at a higher magnification; HE = 200×. (D) Photomicrograph of the bursa of Fabricius of a commercial poult inoculated at 3 days of age with turkey-origin reovirus (TRV) isolate NC/SEP-R44/03 (SEP/44), at 7 days postinoculation. There is both cortical and medullary follicle lymphoid depletion and stromal fibroplasia; HE = 200×.

previous study was also noted in lymphocytes that apparently had no virus present (11); this could be another mechanism for the decreased lymphoproliferation noted following mitogen injection. The ability of different strains of chicken reovirus and the purified reovirus cell attachment protein σC to cause apoptosis in avian and mammalian cell culture has also been demonstrated (7,18). The CBH response in 3-week-old commercial poults was more difficult to measure directly, due to the size of the birds and their toe webs at that age, but it was not significantly reduced in infected poults. Based, in part, upon our experience, the interdigital skin test appears to be better suited to younger birds (2).

Based upon the marked bursal atrophy observed in SPF (11,20) and commercial poults, the effect of TRV infection on the humoral immune response was not a surprise. The ability of TRV-infected poults to produce antibodies to NDV was markedly diminished at the 21-day post-NDV exposure time point; this inhibition was less striking at 42 days post-NDV exposure. The inhibition of the humoral immune response also appears to be age-dependent, as the TRV-infected 3-week-old commercial poults revealed no significant reduction in their ability to mount an antibody response at 21 and 42 days postexposure. Similar studies in reovirus-infected chickens did not result in a decrease in the antibody response to NDV; however, the extent of reovirus-induced lymphoid depletion in the bursa of Fabricius was not as severe as that noted in poults at approximately the same age (8,9). The extent of the damage noted in the bursas of Fabricius of infected poults at 8 and 15 DPI can result in permanent immunosuppression, a state of particular importance for a virus often associated with syndromes such as PEC that involve multiple viral and perhaps bacterial agents, particularly when coupled with an impaired cell-mediated response that may be unable to clear virus-infected cells properly. Further, the age-related differences noted in the NDV-exposed poults suggest that the timing of exposure to an immunosuppressive agent like TRV may be important for the development of recognizable and consequential enteric syndromes in the field. The reovirus-induced immune dysfunction in both the humoral and cell-mediated branches of the poult immune system may implicate certain strains of TRV as the first step in one of several paths to enteric disease.

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